DEVELOPMENT OF CHITOSAN/ALGINATE/SILVER NANOPARTICLES HYDROGEL SCAFFOLD FOR SOFT TISSUE ENGINEERING APPLICATIONS

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A thesis submitted in
fulfillment of the requirement for the award of the
Degree of Master of Mechanical Engineering with Honours

Faculty of Mechanical and Manufacturing Engineering
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AUGUST 2017

This thesis is dedicated to

My father, Ramli B. Mohammed

My mother, Rosiyah Bt. Suratman

Husband, Muhammad Basry Bin Mahsun

Little caliph in my womb that go through this thesis writing and project with me and safely born during my thesis writing period, Muhammad Haqeem Naufal Bin Muhammad Basry

My siblings, Roslina Hanim Ramli, Rafizul Hafiz Ramli and Rafirul Hanif Ramli

My family in-laws

My Project Supervisor and Co-Supervisor, Associate Professor Dr. Anika Zafiah Mohd Rus and Associate Professor Dr. Soon Chin Fhong

And all my colleagues and friends.

LIST OF ASSOCIATED PUBLICATIONS

Conference Papers

1. Synthesis of Chitosan/Alginate/Silver Nanoparticles Hydrogel Scaffold. Ramli, R. H., Soon, C. F., & Rus, A. Z. M. (2016). Green Design and Manufacture Internationaal Conference, Phuket, 1-2 May 2016. In *MATEC Web of Conferences* (Vol. 78, p. 01031). EDP Sciences.
2. Characterisation of silver nanoparticles produced by three different methods based on Borohydride reducing agent. Ramli, R. H., Soon, C. F., & Rus, A. Z. M. (2016). Green Design and Manufacture Internationaal Conference, Phuket, 1-2 May 2016. In *MATEC Web of Conferences* (Vol. 78, p. 01032). EDP Sciences.

ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah that I finally able to finish my master's study. First of all, I would like to express my sincere appreciation to my supervisor and co supervisor, Associate Prof. Dr. Anika Zafiah Mohd. Rus and Associate Prof. Dr. Soon Chin Fhong for the support given throughout the duration for this research. Without their continuous support and patience throughout this research and thesis writing, I will not be able to complete it on time.

I also would like to express my appreciation especially to MyBrain15, Universiti Tun Hussein Onn Malaysia and GIPS Vot U337 for funding me financial support during the study. Many thanks to all my colleagues in this university that always help me and provide any support throughout this research, Arina Basyirah, Nurul Hamizah, Wong and Leong. I would also like to thank to the technicians involved in this research, Madam Faezahana, Miss Isrihetty and Mr. Fazlan.

In addition, I would like to thank to Microelectronics and Nanotechnology-Shamsudin Research Centre (MiNT-SRC) and Faculty of Mechanical Engineering (FKMP) of UTHM for providing the facilities for me to conduct this research. Appreciation also goes to everyone involved directly or indirectly towards the compilation of this thesis. Last but not least, author would specially thank to beloved parents, husband, families and friends. Without their moral support, author will not have the courage to continue the study and completed the thesis.

ABSTRACT

A biodegradable scaffold in tissue engineering serves as a temporary skeleton to accommodate and stimulate new tissue growth. Alginate (Alg) and chitosan (Chi) are both popular materials applied as biomaterials or bioimplants. However, Alg derived from brown algae is highly compliant and easily decomposed in fluid, whilst Chi derived from shrimp shells has weak strength. In rectify these problems, the development of Chi and Alg based biodegradable scaffolds incorporated with silver nanoparticles (AgNPs) with enhanced mechanical properties and biosafe function is proposed. Different ratios of chitosan/alginate (Chi/Alg) were prepared and the effect of different ratio (1:1, 1:2 and 2:1) to the mechanical, biological properties with and without AgNPs and keratinocyte cell growth were investigated. The preliminary result of FTIR, UV-Vis, XRD, FESEM and EDS proved the production of silver nanoparticles. Meanwhile, FTIR, swelling/degradation, DMA, TGA, DSC, FESEM and MTT assay was conducted to study the properties of Chi/Alg based scaffold. FTIR analysis shows the crosslinking of Chi/Alg based scaffold. Swelling/degradation and DMA shows Chi/Alg and chitosan/alginate/silver nanoparticles (Chi/Alg/AgNPs) has adequate swelling and compressive modulus that exceed the epidermis' Young modulus, thus able to provide mechanical support upon application. Meanwhile, the thermal analysis revealed that the onset decomposition temperature of scaffold were at around 70 ºC which is due to the loss of water present in the scaffold thus thermally safe for soft tissue application. Based on FESEM result, there are different in surface structure of Chi/Alg based scaffold. Finally, with the incorporation of 0.3 % PVP synthesised AgNPs in Chi/Alg based scaffold, cells are able to live up to 14 days. As a result, Chi incorporation in the Alg and AgNPs improved physical, mechanical properties of hydrogel itself and provide biosafe environment during the study.

ABSTRAK

Di dalam kejuruteraan tisu, perancah atau acuan biodegradasi berfungsi sebagai struktur sementara untuk menampung dan merangsang pertumbuhan tisu baru. Antara bahan yang sering digunakan sebagai bio-bahan serta bio-implant adalah alginate (Alg) dan chitosan (Chi). Namun begitu, walaupun sifat Alg dan Chi yang kedua-duanya berasal dari bahan organik iaitu alga coklat dan kulit udang, Alg sangat mudah melarut di dalam cecair manakala Chi mempunyai kekuatan yang rendah. Maka, penggabungan Chi dan Alg serta nanopartikel perak (AgNPs) bagi membentuk perancah biodegradasi dilakukan untuk meningkatkan sifat mekanikal serta fungsi keselamatan biologi. Chitosan/alginate (Chi/Alg) dengan berbeza nisbah (1: 1, 1: 2 dan 2: 1) disediakan sama ada dengan penambahan atau tanpa penambahan AgNPs dan kesannya terhadap sifat mekanikal, biologi serta pertumbuhan sel keratinocyte telah disiasat. Keputusan awal FTIR, UV-Vis, XRD, FESEM dan EDS membuktikan penghasilan AgNPs. Manakala ujian FTIR, degradasi, DMA, TGA, DSC, FESEM dan MTT assay telah dijalankan untuk mengkaji sifat-sifat perancah biodegradasi Chi/Alg. Analisis FTIR menunjukkan Chi/Alg telah bergabung. Keputusan kadar pengembangan di dalam cecair dan DMA menunjukkan Chi/Alg dan Chi/Alg/AgNPs mempunyai kadar pengembangan yang seimbang dan modulus mampatan yang melebihi mampatan epidermis kulit dan seterusnya mampu member sokongan mekanikal sewaktu aplikasi penggunaan. Sementara itu, terma analisis mendedahkan bahawa suhu permulaan penguraian perancah berada di sekitar 70 ° C yang disebabkan oleh kehilangan air di dalam perancah, maka ia selamat digunakan. Berdasarkan keputusan FESEM, permukaan Chi/Alg juga berbeza. Tambahan lagi, penambahan 0.3 % PVP AgNPs dalam perancah Chi/Alg tidak memberikan kesan kepada sel kerana sel mampu untuk hidup sekurang-kurangya selama 14 hari. Kesimpulannya, gabungan Chi dalam Alg dan AgNPs telah meningkatkan sifat fizikal, sifat mekanik hidrogel sendiri dan menyediakan persekitaran biologi yang selamat semasa kajian.

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LIST OF ABBREVIATIONS

DMA	-	Dynamic Mechanical Analysis
FTIR	-	Fourier Transform Infrared Spectroscopy
DSC	-	Differential Scanning Calorimetry
TGA	-	Thermogravimetric Analysis
AFM	-	Atomic Force Microscopy
SEM	-	Scanning Electron Microscopy
PEG	-	Polyethylene glycol
DI water	-	Deionized water
PLA	-	Poly(lactic acid)
PGA	-	Poly(glycolic acid)
PLGA	-	Poly(lactic-co-glycolic acid)
AgNPs	-	Silver nanoparticles
Chit	-	Chitosan
Alg	-	Alginate

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CHAPTER 1

INTRODUCTION

1.1 Research background

Every year, a large number of individuals are suffering from tissue impairment and organ malfunction due to the accident and illness (Adhikari *et al*., 2016). This impaired tissues and aged cells are restored by the self-healing ability of human body. However, the restore capacity of these mature tissues is frequently inadequate if the injury caused severe damage (Upadhyay, 2015). Hence, synthetic devices, donor organs as well as autologous transplants are utilised to substitute unfixable harmed tissues and organs. However, these procedures failed to substitute the organ failure completely. Thus, the development of alternative strategies was caused by demand for transplantation of organs and tissue exceeding the supply (Rouchi & Mahdazvi-Mazdeh, 2015). Additionally, Ministry of Health (Malaysia) communicated that, up to 31 January 2015, practically 19 353 patients require organ transplantation and the organ transplantation rate in Malaysia is the lowest in the world which is 0.68 donor for every one million people. This issue is caused by the supply which is the donor organ that unable to fulfil the demand which is the increasing number of patients (Kementerian Kesihatan Malaysia, 2017).

At regular intervals based on U.S. Department of Health & Human Services, transplant waiting list is expanding and averagely 21 patients die while waiting for the transplantation (U.S. Department of Health & Human Services, 2016). The deficiency of organs or effective organ replacement has caused thousands die every year while waiting for transplantation. Approaches such as substituting impaired organ through mechanical approach (heart lung bypass machines and dialysis) and artificial devices (joint substitution) are short-term solutions used by the clinicians.

However, these approaches do not allow the patients to completely recover from their injury and may have several limitations to conduct their habitual activity. These methods also may cause the state of being infected and refusal act of the body to the foreign device upon transplantation (Orlando *et al*., 2013). These issues need to be addressed by new development in the area of tissue engineering. Research on designing manufactured biomaterials that can supplant harmed or injured tissue for short-term and long-term substitution was conducted due to the necessity for substituting damaged tissue in human body (Park, 2011).

Biomaterial is any biocompatible material, natural or synthetically, that is used to supplant or part of an organ or its tissue, while in private contact with living tissue (Chen & Thouas, 2014). Biomaterials moreover defined as any substance (other than a prescription) or blend of substances, originated naturally or synthetically, which can be used for any time period, with clinical trials taken into account or as a part of a structure which treats, extends, or replaces any tissue (Censi, 2010). Hydrogel frameworks are one of various kinds of biomaterials that has been utilised broadly as part of tissue building applications (Park, 2011).

Due to its favorable characteristics, hydrogels have been applied widely in biomedical such as tissue engineering as well as drug delivery. Their high water content renders them great with living tissues and proteins and their viscoelasticity minimises damage to the encapsulating tissue. Hydrogels are commonly appealing in field of tissue engineering due to their mechanical properties alike to the natural tissues. The biodegradability properties of hydrogels permit the hydrogels transplantation into human body, without the need of second surgery after the hydrogel degraded in the body because the extracellular matrices by the incorporated cells after a period of time will replace the degraded hydrogel (Zhao *et al*., 2015, Chai *et al*., 2017).

In this project, chitosan/alginate based hydrogel with the inclusion of silver nanoparticles was proposed for application as a bio-scaffold in tissue engineering. Chitosan and alginate are natural occurring polymers and offer several advantages to mimic natural extracellular matrix (ECM) in the body. However, these two materials presented have certain drawbacks when used independently (Lanza, 2011; Kim, 2013; Szymanska, 2015). Silver nanoparticles were reported with antibacterial properties in past research (Thomas, 2015). When chitosan and alginate are mixed and incorporated with silver nanoparticles, it is believed that the produced hydrogel

composites scaffold will improve the mechanical properties, biocompatibility, biodegradability and cell growth during the *in-vitro* study as well provide biosafe environment for the growth of seeded human keratinocyte cell lines.

1.2 Problem statement

Alginate and chitosan has been utilised broadly as hydrogel framework either separately or by blended with different materials, for example, hyaluronidase enzyme core-5-fluorouracil-loaded chitosan/PEG/gelatine polymer nanocomposites (Rajan *et al.*, 2013), Chitosan-nanohydroxyapatite (Roy & Sailaja, 2015), Polycaprolactone-alginate (PCL-alginate) (Kim & Kim, 2015), and hydroxyapatite/chitosan-alginate (Han *et al*., 2010) composite scaffolds for applications in tissue engineering. Chitosan has been broadly utilised as a part of the composites blended with collagen, coral and hydroxyapatite to grow new frameworks for tissue designing applications, however the mix of chitosan/alginate has not been much investigated (Lanza *et al*., 2011). The major disadvantages of using chitosan alone as biopolymer are its weak physical strength (Kim, 2013), high swelling tendency (Agarwal & Murthy, 2015) and the restricted solubility of chitosan (Kim, 2013).

In overcome these problem, some chemical modification of chitosan either on $–NH_2$ groups of glucosamine units or on –OH groups of the polymer, cross-linking and the incorporation with other materials was conducted to improve its properties to mimic the structure of the tissue (Saikia *et al*., 2015; Aryaei, 2014; Jayakumar *et al.*, 2011, Rodríguez-Vázquez *et al*., 2015). However, chitosan was found as an attractive natural biopolymer as it resembles glycosaminoglycan (GAGs) structure which is the main component of extracellular matrix (ECM) and its hydrophilic nature aids in cell adhesion, proliferation as well as differentiation. Besides that, chitosan also has aggregated polymeric chains which are compact thus provide stability to the scaffold in terms of application to be used in tissue engineering (Jayakumar *et al.*, 2011). In contrast, despite of being non-immunogenic, biocompatible and gentle gelling properties of alginate, alginate will be exposed to high degradability when exposed to fluid. The loss of divalent cations from alginate to the surrounding medium is uncontrollable, thus it caused hydrogel with unpredictable degradability ability, limited stability for long term effect as well as limited strength and toughness that

mainly depends on alginate's chemical structure (G content effect on stiffness) (Lee & Mooney, 2012; Sun & Tan, 2013).

In this research study, the development of chitosan/alginate hydrogel biocomposites for soft tissue engineering application mainly to improve the mechanical properties of the produced hydrogel itself will be conducted. This biocomposite with properties that complements each other is expected to improve mechanical and biophysical properties of the hydrogels. Silver nanoparticle is a non-toxic material and highly effective for antimicrobial activities. An addition of silver nanoparticles to the chitosan/alginate composites would create a compatible environment for the growth of seeded human keratinocyte cell lines.

1.3 Hypothesis of the research

The development of chitosan based hydrogel as natural based scaffold shows tremendous growth in the tissue engineering field. The disadvantage of chitosan in tissue engineering scaffolds is its limited solubility (Kim, 2013), stability and weak mechanical properties with range of 2.5 kPa modulus at 0.4 wt % of chitosan hydrogels (Mushi, 2014). Chitosan is an attractive natural biopolymer as it resembles GAGs of ECM that enhance the cell adhesion, proliferation and also differentiation to be used in tissue engineering application. However, chitosan has weak mechanical properties, limited solubility and stability (Kim, 2013). In contrast, even though alginate has simple gelling ability, biocompatible and non-immunogenic properties, alginate's drawback such as unpredictable degradability due to loss divalent cations and mechanical properties that depends on chemical structure of alginate has brought the effort to incorporate alginate with other materials to overcome its drawback (Lee & Mooney, 2012; Sun & Tan, 2013). Thus, by incorporating these two materials, chitosan/alginate hydrogel were believed to enhance the biocompatibility, solubility, and mechanical properties of the biocomposite to be used in soft tissue engineering application. Silver nanoparticles further added to improve the properties of hydrogel while ensuring the hydrogel is biosafe to be applied as implants later.

1.4 Aim of research

The aim of this research is to develop renewable source for damaged tissue with biocompatible, biodegradable and tunable mechanical properties. In this work, hydrogel scaffolds by using natural (chitosan & alginate) polymer derivatives with incorporation of silver nanoparticles were synthesised and characterised for tissue engineering applications.

1.5 Research objective

i. To synthesise silver nanoparticles (AgNPs).
ii. To synthesise chitosan/alginate hydrogel scaffolds with incorporation of silver nanoparticles.
iii. To characterise physical and mechanical properties of chitosan/alginate hydrogel with/without inclusion of silver nanoparticles.
iv. To investigate the effect of an addition of silver nanoparticle in chitosan/ alginate with the seedings of human keratinocyte cell lines (HaCaT).

1.6 Scope of research

This research focused on developing hydrogel scaffold by mixing different biopolymers with different ratio and examining the mechanical and biophysical properties of the chitosan/alginate scaffold with an inclusion of silver nanoparticles. The silver nanoparticles also synthesised based on previous research (Malina *et al*., 2012) and applied in this work. Functional groups of the scaffold were studied using Fourier Transform Infrared Spectroscopy (FTIR). While mechanical properties of materials were measured by Dynamic Mechanical Analyses (DMA). Thermal stability and decomposition of the hydrogel were conducted by Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) respectively. Molecular surface structures were investigated by using Field Emission Scanning Electron Microscopy (FESEM). Apart from that, cell viability was studied by using MTT assay analysis. Swelling and degradation of the produced hydrogel were also being investigated.

1.7 Thesis contribution

In this dissertation, biosafe chitosan/alginate composites with the incorporation of silver nanoparticles finally produced. The characterisation of chitosan/alginate and chitosan/alginate/silver nanoparticles by different methods has been investigated and the main contribution of this research is by investigating the effect of addition of silver nanoparticles into chitosan/alginate based biocomposites to the human skin cells seeding as well as the study of its mechanical properties that has not been explored before. This result can be further used for other applications of tissue engineering.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Chapter 2 presented the literature studies on tissue engineering, especially in soft tissue engineering (Section 2.2). In this sub section, the literature studies presented the history of tissue engineering, the approach and the materials that were incorporated in the area. The structure of skin and human keratinocytes were discussed in Section 2.3. Section 2.4 presented the physical and chemical properties of hydrogels and natural extracellular matrix (ECM) while Section 2.5 and 2.6 discussed on the natural biomaterials and synthetic biopolymers respectively. Section 2.7 meanwhile elaborated the biomaterials used in tissue engineering which chitosan (Section 2.7.1) and alginate (Section 2.7.2). Next, silver nanoparticles and its mechanism on antibacterial were discussed on Section 2.8. The mechanical and physical characterisation techniques such as thermal analysis, chemical analysis, mechanical analysis and electron microscopy were discussed in Section 2.9. And finally the summary of literature review is discussed on Section 2.10.

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